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RESEARCH ARTICLE

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Development of cerebral mitochondrial respiratory function is impaired by thyroid hormone deficiency before birth in a region-specific manner

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Abstract

Thyroid hormones regulate adult metabolism partly through actions on mitochondrial oxidative phosphorylation (OXPHOS). They also affect neurological development of the brain, but their role in cerebral OXPHOS before birth remains largely unknown, despite the increase in cerebral energy demand during the neonatal period. Thus, this study examined prepartum development of cerebral OXPHOS in hypothyroid fetal sheep. Using respirometry, Complex I (CI), Complex II (CII), and combined CI&CII OXPHOS capacity were measured in the fetal cerebellum and cortex at 128 and 142 days of gestational age (dGA) after surgical thyroidectomy or sham operation at 105 dGA (term ~145 dGA). Mitochondrial electron transfer system (ETS) complexes, mRNA transcripts related to mitochondrial biogenesis and ATP production, and mitochondrial density were quantified using molecular techniques. Cerebral morphology was assessed by immunohistochemistry and stereology. In the cortex, hypothyroidism reduced CI-linked respiration and CI abundance at 128 dGA and 142 dGA, respectively, and caused upregulation of *PGC1α* (regulator of mitochondrial biogenesis) and thyroid hormone receptor β at 128 dGA and 142 dGA, respectively. In contrast, in the cerebellum, hypothyroidism reduced CI&II- and CII-linked respiration at 128 dGA, with no significant effect on the ETS complexes. In addition, cerebellar glucocorticoid hormone receptor and adenine nucleotide translocase (*ANT1*) were downregulated at 128 dGA and 142 dGA, respectively. These alterations in mitochondrial function were accompanied by reduced myelination. The findings demonstrate the importance of thyroid hormones in the prepartum maturation of cerebral mitochondria and have implications for the etiology and treatment of the

Abbreviations: ANT1, adenine nucleotide translocase 1; BSA, bovine serum albumin; CS, Citrate synthase; DAB, 3,3′-Diaminobenzidine; dGA, days of gestational age; DRP1, dynamin-related protein 1; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ETS, electron transfer system; GI, gyrification index; GR, glucocorticoid receptor; H_2O_2, hydrogen peroxide; MBP, myelin basic protein; MFN2, mitofusin 2; OD, optical density; OXPHOS, oxidative phosphorylation; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; T_3, triiodothyronine; T_4, thyroxine; *THRβ*, thyroid hormone receptor beta; TX, thyroidectomized; UCP2, uncoupling protein 2.

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neurodevelopmental abnormalities associated with human prematurity and congenital hypothyroidism.

KEYWORDS

brain, fetus, mitochondria, thyroid hormones

1 | INTRODUCTION

Congenital hypothyroidism affects 1 in 2000 neonates worldwide.[1] It is associated with prolonged gestation, abnormal birth weight, and impaired neurological development.[1-3] Even with postnatal hormone therapy, these infants are at greater risk of cognitive delay, lower intelligence, and deficits in fine motor control in later childhood.[2,4] Similarly, neonatal subclinical hypothyroidism is associated with delayed attainment of the normal neurodevelopmental and educational milestones.[4,5] Recent studies have also indicated that the effects of fetal hypothyroidism on human brain development may extend beyond the psychomotor to include central regulation of metabolism in later life.[5]

In mammals, thyroid hormones stimulate tissue accretion and differentiation *in utero*, and have a key role in maturation of tissues essential for immediate neonatal survival, such as the lungs and liver.[6] Perinatal hypothyroidism also adversely affects neuronal development of specific brain regions in newborn rodents and sheep.[2,7] In adulthood, thyroid hormones have a central role in controlling thermogenesis and metabolic rate, in part, through actions on mitochondrial oxidative phosphorylation (OXPHOS).[8] At birth, energy demands increase for new postnatal processes, such as thermogenesis, muscular activity, and metabolic homeostasis, in association with a rise in oxygen consumption.[6,9] Hypothyroidism is known to limit this rise in oxygen consumption and impair thermogenesis in newborn lambs.[10,11] More recent studies have also shown that thyroid hormones are involved in the normal perinatal maturation of mitochondrial OXPHOS capacity in metabolic tissues including liver, skeletal muscle, and adipocytes.[12-14] However, relatively little is known about the role of thyroid hormones in cerebral mitochondrial development,[15] despite the high energy demands associated with the increased neuronal differentiation and activity during the perinatal period.[16]

In adult tissues, thyroid hormones regulate many aspects of mitochondrial function including the abundance of the electron transfer system (ETS) complexes generating the electrochemical proton gradient driving ATP production, the uncoupling proteins (UCPs) dissipating this gradient, and the adenine nucleotide translocases (ANTs) that transports ADP into and ATP out of the mitochondria.[17] Thyroid hormones also affect mitochondrial biogenesis, fusion, and fission that control the density, dynamics, and functional organization of the mitochondrial membranes.[2,18] Recent studies of intrauterine hypothyroidism have shown that thyroid hormones can influence several of these processes in metabolically active fetal tissues such as adipocytes and skeletal muscle.[13,14,18,19] However, little is known about the effects of thyroid hormone deficiency on the factors regulating mitochondrial OXPHOS efficiency in the fetal brain near term.

This study examined mitochondrial function of the cortex and cerebellum of thyroidectomized fetal sheep during late gestation in relation to expression of genes and proteins involved in mitochondrial biogenesis, electron transport, and OXPHOS efficiency. Sheep were chosen for this study because of their similarity to human infants in terms of the autonomy of the fetal hypothalamic-pituitary-thyroid axis in late gestation and of the timing of key developmental events in the brain compared to more altricial rodent species.[6,7] The study tested the hypothesis that thyroid hormone deficiency would impair mitochondrial OXPHOS of the brain before birth, in association with altered cerebral morphology.

2 | MATERIALS AND METHODS

2.1 | Animal experimental procedures

All experimental procedures were carried out under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (Licence Number: PC6CEFE59), after ethical approval by the Animal Welfare and Ethical Review Body of the University of Cambridge, UK.

Nineteen time-mated Welsh mountain ewes with twin pregnancies were group housed with free access to hay and water. Between 102 and 105 days of gestational age (dGA; term ~145 dGA), 13 ewes were anesthetized (1.5%-2.0% isoflurane in O_2:N_2O) after an overnight fast and one fetus was surgically thyroidectomized (TX), while its twin was sham operated as a control as described previously.[13] Treatment was randomized by alternating fetal TX between the right and left uterine horn in successive ewes. At surgery, ewes received analgesia (1 mg/kg carprofen, sc.) and antibiotics (oxytracyclin, 20 mg/kg im.); both ewe and fetuses received penicillin (15 mg/kg Depocillin im to mother and 600 mg Crystapen intra-amniotically to fetus). Maternal penicillin treatment was continued for 2 days post-surgery.

At either 125-131 dGA (n = 7) or 140-145 dGA (n = 6), operated ewes and their fetuses were euthanized for tissue

collection with a lethal dose of anesthetic (200 mg/kg sodium pentobarbitone, iv.). The fetuses were delivered by caesarean section in a random order and a blood sample taken from the umbilical artery before final euthanasia. They were weighed and measured before collecting the brain and other tissues. Blood samples were centrifuged and the plasma was stored at −20°C for subsequent hormone analyses. TX fetuses had no thyroid remnants. In addition, between 104 and 105 dGA, fetal tissues were collected from an additional six unoperated twin-bearing ewes after maternal and fetal euthanasia as described above. Only one twin of each of these control pairs was selected randomly for subsequent analyses. Sample size was 4-7 fetuses per age and treatment group and was based on published respirometry data on mitochondrial dysfunction in skeletal muscle of fetal sheep.[13]

The brains were hemisected; fresh tissue samples from the cerebellum and frontal cortex of the cerebrum were collected from the left hemisphere and placed in ice cold buffer (miR05; pH7.1 solution containing 0.5 mM EGTA, 3 mM magnesium chloride, 60 mM K-lactobionate, 20 mM taurine, 10 mM potassium phosphate monobasic, 20 mM HEPES, 110 mM sucrose, and 0.1% (w/v) bovine serum albumin) for respirometry. The remaining portions of the frontal cortex and cerebellum of the left hemisphere were frozen in liquid nitrogen and stored at −80°C for subsequent molecular analysis. The right hemisphere was immersion fixed in 4% paraformaldehyde (PFA) for stereological analysis.

2.2 | Plasma hormone concentrations

Umbilical plasma (triiodothyronine) T_3 and (thyroxine) T_4 concentrations were measured by radioimmunoassay (MP Biomedicals, Loughborough, UK). The inter- and intra-assay coefficients of variations were 8% and 2%, respectively, for T_3, 5%, and 3% for T_4. The minimum levels of detection were 0.14 ng/mL for T_3 and 11.3 ng/mL for T_4. Plasma cortisol concentrations were measured using an ELISA kit (IBL International, Hamburg, Germany). The inter- and intra-assay variations were 5% and 3%, respectively, and the minimum level of detection was 5.2 ng/mL. Concentrations measured at, or below, the limit of detection of the assay were assigned the minimum value of detection for that hormone.

2.3 | Protein and water content quantification

Total protein was extracted from frozen cerebellar and cortical samples. Samples were homogenized with lysis buffer (pH7.5 solution containing 20mM Trizma-hydrochloride, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mMNa pyrophosphate, 1mM β-glycerophosphate, and 1mM Na orthovanadate, Sigma Aldrich, UK) and protein concentration determined using a bicinchoninic acid assay (Sigma Aldrich, UK), expressed as mg protein per gram wet weight. Weighed cerebellar and cortical samples were freeze-dried for 24 hours to measure water content, which was then used to calculate percentage dry weight.

2.4 | Citrate synthase activity assay

Citrate synthase (CS) is commonly used as a putative marker of mitochondrial density.[20,21] Its activity was measured in 20 μg of the homogenized cerebellar and cortical protein samples by a spectrophotometric enzyme assay. The assay buffer contained 0.1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 1 mM oxaloacetate, and 0.3 mM acetyl-CoA (pH 8). Cerebral CS activity was determined from the maximum rate of change in absorbance at 412 nm and 37°C (rate of thionitrobenzoic acid (TNB) production) over a 3-minute period, and expressed as μmoles per minute per mg protein.

2.5 | Mitochondrial respiratory function

High-resolution respirometry was used to assess mitochondrial OXPHOS rates using a range of substrates that provide electrons to the ETS through different ETS complexes. Samples (~10 mg) from the cortex (at the level of the ansate sulcus) and cerebellum (at the level of the horizontal fissure) were homogenized in miR05 and transferred into oxygraph chambers (Oxygraph 2k, Oroboros Instruments, Austria). Oxygen concentration (μM) and flux per tissue mass (pmol O_2/s/mg) were recorded in real-time using calibrated oxygen sensors and Datlab software (Oroboros Instruments, Austria). Respiratory rates were corrected for instrumental background by DatLab, taking into account oxygen consumption of the oxygen sensor and oxygen diffusion out of or into the oxygraph chamber measured under experimental conditions in miR05 medium without any tissue sample. A substrate-inhibitor titration protocol was performed.[22] Non-phosphorylating leak respiration (CI_L) was induced by adding the Complex I (CI)-linked substrates pyruvate (5 mM) and malate (2 mM). ADP (10 mM; saturating concentration) was added to stimulate OXPHOS. OXPHOS capacity of CI-linked substrates (CI_P) was achieved by addition of glutamate (10 mM, saturating concentration). Maximization of electron flux through Complex I and Complex II was achieved through addition of CII-linked substrate, succinate (10 mM, $CI\&CII_P$). Subsequently, inhibition of CI by rotenone (0.5 μM) provided measurement of CII-linked OXPHOS capacity (CII_P). Cytochrome c (10 μM) was added to check the integrity of the mitochondrial membranes with data excluded if respiration increased by >15%. Respiration rates were corrected for dry weight (see Section 2.3).

The fraction of OXPHOS capacity dissipated in the leak state was calculated as follows:

$$C_L/CI\&CII_P.$$

OXPHOS coupling efficiency was calculated for the CI-linked substrates, represents the net OXPHOS capacity, and corrected for leak respiration (CI_L):

$$\left[CI_P - CI_L\right]/CI_P = 1 - CI_L/CI_P.$$

Flux control ratios were calculated to discern the fraction of OXPHOS capacity attributable to CI- and CII-linked respiration as follows:

$$CI_P/CI\&CII_P \text{ (flux control ratio of Complex I)}.$$

$$CII_P/CI\&CII_P \text{ (flux control ratio of Complex II)}.$$

2.6 | Western blotting

The protein abundance of ETS complexes (CI-CIV) and ATP synthase were determined using Western blotting. In brief, total protein was extracted from frozen cerebellum and cortex samples (~50 mg), diluted to 2.5 μg/μl in 8% SDS solution, and separated by electrophoresis on a 12% polyacrylamide gel. Protein was transferred to a nitrocellulose membrane then stained with Ponceau-S to normalize for protein loading. Membranes were probed with an antibody cocktail to ETS complexes (OXPHOS antibody cocktail; Life Technologies, Carlsbad, CA, USA; 458099; 1:1000; RRID: AB_2533835) followed by HRP-linked secondary antibody (GE Healthcare, Amersham, UK; NIF82; 1:5000). Protein bands were visualized using enhanced chemiluminescence, then quantified using ImageJ (NIH, Figure S1).

2.7 | Gene expression analyses

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine changes in the expression of genes involved in the regulation of mitochondrial biogenesis and dynamics. Frozen samples from the cerebellum and cortex were homogenized in Lysing Matrix-D tubes (MP Biomedicals, Loughborough, UK) using a MagNA Lyser (Roche Diagnostics, Almere, The Netherlands). The RNA was extracted (RNeasy Plus Mini Kit, Qiagen, Manchester, UK) and yields assessed using a Nanodrop (Thermo Fisher Scientific, Loughborough, UK). Reverse transcription of the extracted mRNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK). Quantitative reverse transcription polymerase chain reaction was performed using MESA BLUE Mastermix (Eurogentec, Seraing, Belgium) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the recommended protocol: an initial denaturation step (5 minutes at 95°C) followed by 40 amplification cycles (15 seconds at 95°C and 1 minute at 60°C). Separate plates were used to measure mRNA levels for each gene and each brain region. Each sample was measured in triplicate. The primer sequences are given in Table S1. In order to compare mRNA abundance of target genes between the treatment groups, cycle thresholds (Ct) were analyzed using the delta-delta-Ct (ddCt) method. The results were expressed as fold changes relative to the mean of the control group at 128 dGA (control vs TX).

2.8 | Brain histology and stereology

The right cerebrum was cut coronally into 5-mm-thick blocks, processed and embedded in paraffin. Sections of 10 μm thick were cut from three blocks (Leica RM 2235 microtome, Germany; Figure 5). The cerebellum was bisected at the midline of the vermis, and the right side of the cerebellum was processed to paraffin wax and sagittally sectioned at 10 μm. Sections from both regions were stained with hematoxylin and eosin (H&E) to assess general morphology.

Due to the critical role of thyroid hormone in regulating axonal myelination,[23] immunostaining of myelin basic protein (MBP) was performed in both brain regions. Briefly, sections were washed in phosphate buffered saline (PBS, Sigma-Aldrich, UK), and then incubated with 3% hydrogen peroxide (H_2O_2). Nonspecific binding was blocked with 4% bovine serum albumin (BSA) in PBS, after which sections were incubated with MBP overnight (1:400 in 2% BSA and 0.3% Triton in PBS; Millipore and Sigma Aldrich, UK). On the following day, sections were incubated with secondary antibody (1:400 in 2% BSA in PBS, Vector Laboratories, USA), and then Avidin-Biotin (Vector Laboratories, UK) in PBS. Staining was visualized by adding metal 3,3′-Diaminobenzidine (DAB, Thermo Fisher Scientific, Loughborough, UK) to the sections. Tissue was then cover slipped with DPX.

To assess total area (H&E) and extent of myelination (MBP immunohistochemistry), scanned sections of each brain region (NanoZoomer, Hamamatsu Photonics, UK) were converted into grayscale and the threshold adjusted using ImageJ (NIH). The optical density (OD) of MBP staining was measured using ImageJ (NIH) and an optical density step tablet. Fields of view within the arbour vitae (cerebellum, n = 10/section), intragyral (cerebrum, n = 10/section), and periventricular white matter (cerebrum, n = 10/section) were sampled. Cortical gyrification was determined using the gyrification index (GI), which describes the degree and complexity of the cortical folding. The GI was calculated as the ratio between the lengths of coronal outlines for the brain including and

excluding the sulcal regions. The lengths were measured on scanned sections using NanoZoomer (Hamamatsu Photonics, UK). All quantitative analyses were performed with the observer (EJC) blind to the treatment groups.

2.9 | Statistics

All values are presented as mean ± SEM. In each brain region, statistical differences from 104 dGA to 142 dGA in control fetuses were assessed by a one-way ANOVA followed by the Tukey's *post hoc* test. Statistical differences between TX and sham-operated control fetuses were determined in each brain region by a two-way ANOVA using treatment (TRT) and age (AGE) as factors followed by Tukey's *post hoc* test, where appropriate, to identify the specific effect of the factors or their interaction (INT). Statistical outliers were detected using the Grubbs test, and any data points falling outside of these criteria were excluded from subsequent analyses. Pearson's correlation coefficient calculation was used to assess linear correlation between variables and log-transformed hormone concentrations. Partial correlation analyses were used to determine the relationship between two variables while controlling for a third variable. P values of $<.05$ were considered significant.

3 | RESULTS

3.1 | Hormone concentrations, biometry, and protein and water content

Thyroidectomy lowered T_3 and T_4 concentrations to close to the minimum detectable values. The normal prepartum rise in plasma T_3 was abolished by thyroidectomy, leading to significantly lower T_3 concentrations in TX than control fetuses by 142 dGA (Table 1). Plasma T_4 concentrations were significantly less in TX than control fetuses throughout, and did not differ with age in either group (Table 1). Consistent with previous findings,[14,24] thyroidectomy attenuated the normal prepartum rise in cortisol concentrations with significantly lower values in TX than control fetuses by 142 dGA (Table 1).

Overall, brain weight and crown rump length were reduced in TX fetuses compared to controls, but increased with age in both groups (Table 1). Body weight and relative brain weight also changed with age in both groups and were not affected by thyroidectomy at either age (Table 1). In both brain regions, protein content increased with age irrespective of treatment but was lower in TX than control fetuses at 128 dGA, although not at 142 dGA (Table 1). Hypothyroidism prevented the prepartum decline in cortical water content seen in the control fetuses, so that by 142 dGA cortical water content was significantly higher in TX fetuses than control values (Table 1). Cerebellar water content was unaffected by hypothyroidism or age (Table 1).

3.2 | Cerebral mitochondrial respiratory function

Cerebral mitochondrial OXPHOS capacity was altered by hypothyroidism in a manner that depended on substrate, gestational age, and brain region (Figure 1). In the cerebellum, thyroidectomy had no significant effect on leak respiration (CI_L) or CI OXPHOS capacity (Figure 1A,B), but reduced $CI\&CII_P$ and CII_P respiration overall relative to control values (Figure 1C,D). In contrast, in the cortex, hypothyroidism significantly reduced CI_L and CI_P respiratory rates at 128 dGA (Figure 1A,B). Cortical $CI\&CII_P$ OXPHOS respiration also tended to be lower in TX than control fetuses at 128 dGA, but this did not reach statistical significance (Figure 1C, $P_{TRT} = .477$, $P_{AGE} = .052$, $P_{INT} = .052$). There was no overall effect of hypothyroidism on cortical CII-linked OXPHOS capacity (Figure 1D). In control fetuses, there were significant increases in $CI\&CII_P$ and CII_P, but not CI_L or CI_P respiration between 104 dGA and 142 dGA in both brain regions (Figure 1C,D). In TX fetuses, cortical CI_L and CI_P respiration increased significantly between 128 dGA and 142 dGA (Figure 1A,B). Hypothyroidism had no effect on CS activity at either age, or on the normal increase in activity seen toward term in either brain region (Figure 1E).

Overall, hypothyroidism had no effect on the fraction of $CI\&CII_P$ OXPHOS capacity related to the leak state, OXPHOS coupling efficiency for CI-linked substrates, or on the fractional contribution of CI to $CI\&CII_P$ OXPHOS capacity, in either brain region (Figure S1A,B,C). However, the contribution of CII to OXPHOS capacity was increased in TX compared to control fetuses at 128 dGA in both brain regions (Figure S1D). Both the fraction of CI&II OXPHOS capacity related to leak, and the contribution of CII to OXPHOS capacity decreased with age in control fetuses in the cortex but not the cerebellum (Figure S1).

When data from all fetuses were combined irrespective of age or treatment (Table 2), cerebellar CI_P and $CI\&CII_P$ respiration rates were correlated positively with T_3 concentrations and, with greater significance, also to cortisol concentrations. Cerebellar CII-linked respiration rates were correlated with cortisol but not T_3 (Table 2). Since T_3 and cortisol concentrations were also correlated ($r = 0.601$, $n = 29$, $P < .001$), partial correlation analysis was used to assess the relative importance of the two hormones. This showed that neither hormone alone was correlated with CI-linked respiration, but that cortisol was the more dominant factor in determining

TABLE 1 Fetal hormonal and biometric measurements

	104 dGA	128 dGA Control	128 dGA TX	142 dGA Control	142 dGA TX	Statistical significance[#]		
	3♀, 3♂	4♀, 3♂	3♀, 3♂	3♀, 3♂	2♀, 4♂	Treatment	Age	Interaction
	103.8 ± 0.5 dGA	128.4 ± 0.8 dGA	128.0 ± 0.8 dGA	142.0 ± 1.0 dGA	142.0 ± 1.0 dGA			
Hormone concentrations								
Plasma cortisol (ng/mL)	13.4 ± 1.0[a]	13.2 ± 1.5[a]	11.7 ± 2.4	52.7 ± 17.0[b #]	22.3 ± 3.7*	$P = .052$	$P = .004$	$P = .077$
Plasma T3 (ng/mL)	0.36 ± 0.02	0.33 ± 0.01	0.23 ± 0.03	0.66 ± 0.16[†]	0.24 ± 0.02*	$P = .002$	$P = .024$	$P = .036$
Plasma T4 (ng/mL)	82.3 ± 9.8	113.1 ± 10.7	13.6 ± 1.1*	96.4 ± 6.5	13.5 ± 1.6*	$P < .001$	$P = .227$	$P = .234$
Morphometry								
Body weight (kg)	1.03 ± 0.06[a]	2.49 ± 0.08[a b]	2.23 ± 0.15	3.46 ± 0.32[b]	3.09 ± 0.2			
Crown-rump length (cm)	31.5 ± 0.7[a]	42.6 ± 0.8[b]	40.2 ± 0.5	45.3 ± 0.7[c]	43.5 ± 1.6[†]	$P = .031$	$P = .004$	$P = .734$
Biparietal diameter (cm)	8.7 ± 0.3[a]	10.6 ± 0.3[b]	12.0 ± 0.7	10.7 ± 0.7[b]	11.0 ± 0.3	$P = .114$	$P = .358$	$P = .304$
Brain weight (g)	24.0 ± 1.2[a]	41.5 ± 1.1[b]	38.2 ± 1.6	47.3 ± 1.9[c †]	43.2 ± 1.6[†]	$P = .018$	$P = .001$	$P = .819$
Brain:body weight ratio (g:kg)	23.4 ± 0.6[a]	16.8 ± 0.5[b]	17.3 ± 0.8	14.0 ± 0.8[c]	14.2 ± 1.0	$P = .637$	$P < .001$	$P = .860$
Biochemical composition								
Cerebellum water content (%)	84.8 ± 1.7	85.6 ± 0.4	86.8 ± 0.7	86.1 ± 0.9	86.4 ± 1.1	$P = .325$	$P = .911$	$P = .557$
Cortex water content (%)	90.2 ± 0.4[a]	88.0 ± 0.9[a b]	88.0 ± 0.5	85.8 ± 0.4[b]	89.0 ± 1.3*	$P = .056$	$P = .454$	$P = .049$
Cerebellum protein content (mg/g)	52.9 ± 8.4[a]	75.6 ± 1.2[a]	65.6 ± 2.1*	77.2 ± 2.7[b]	75.1 ± 2.0[†]	$P = .011$	$P = .018$	$P = .084$
Cortex protein content (mg/g)	40.6 ± 2.2[a]	53.3 ± 2.2[a]	45.4 ± 1.2*	51.6 ± 1.8[b]	52.6 ± 1.4[†]	$P = .046$	$P = .106$	$P = .013$

Note: Data are presented as mean ± SEM from 104-142 days of gestational age (dGA) for control and thyroidectomized (TX) fetuses. For the controls, values with different letters are significantly different from each other ($P < .05$ by a one-way ANOVA + Tukey's *post hoc* test).

[#]Statistical significance for the comparison of control and TX data by two-way ANOVA with treatment and age as factors plus their interaction; *significantly different from control at the same gestational age, and; [†]significantly different from value at 128 dGA ($P < .05$ by two-way ANOVA + Tukey's *post hoc* test).

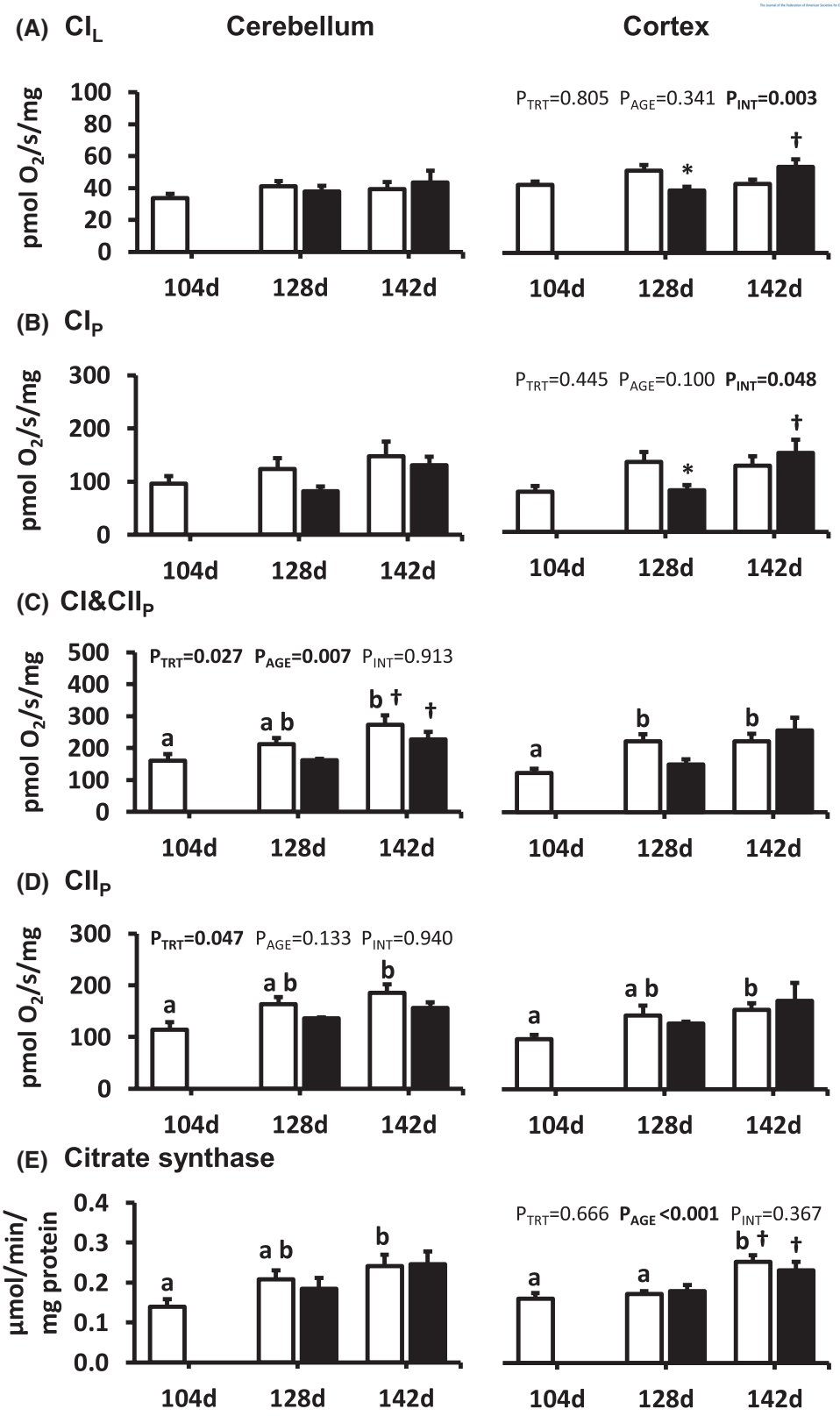

FIGURE 1 Mitochondrial respiratory function in the cerebellum and cortex and impact of hypothyroidism. Mean ± SEM rates of (A) leak respiration (CI_L), (B) Complex I-linked respiration (CI_P), (C) CI&CII-linked respiration ($CI\&CII_P$), (D) CII_P-linked respiration (CII_P), and (E) citrate synthase activity in the cerebellum and cortex from 104 to 142 days (d) gestation for control (open columns) and thyroidectomized (TX, filled columns) fetuses. For the controls, values with different letters are significantly different from each other ($P < .05$ by one-way ANOVA + Tukey's *post hoc* test per region). Statistical significance following a two-way ANOVA examining the effects of treatment (P_{TRT}), age (P_{AGE}), and their interaction (P_{INT}) are presented above each figure when significant, *significantly different from control at the same gestational age, and †significantly different from values at 128 dGA ($P < .05$, two-way ANOVA + Tukey's *post hoc* test per region). n = 4-7 per group

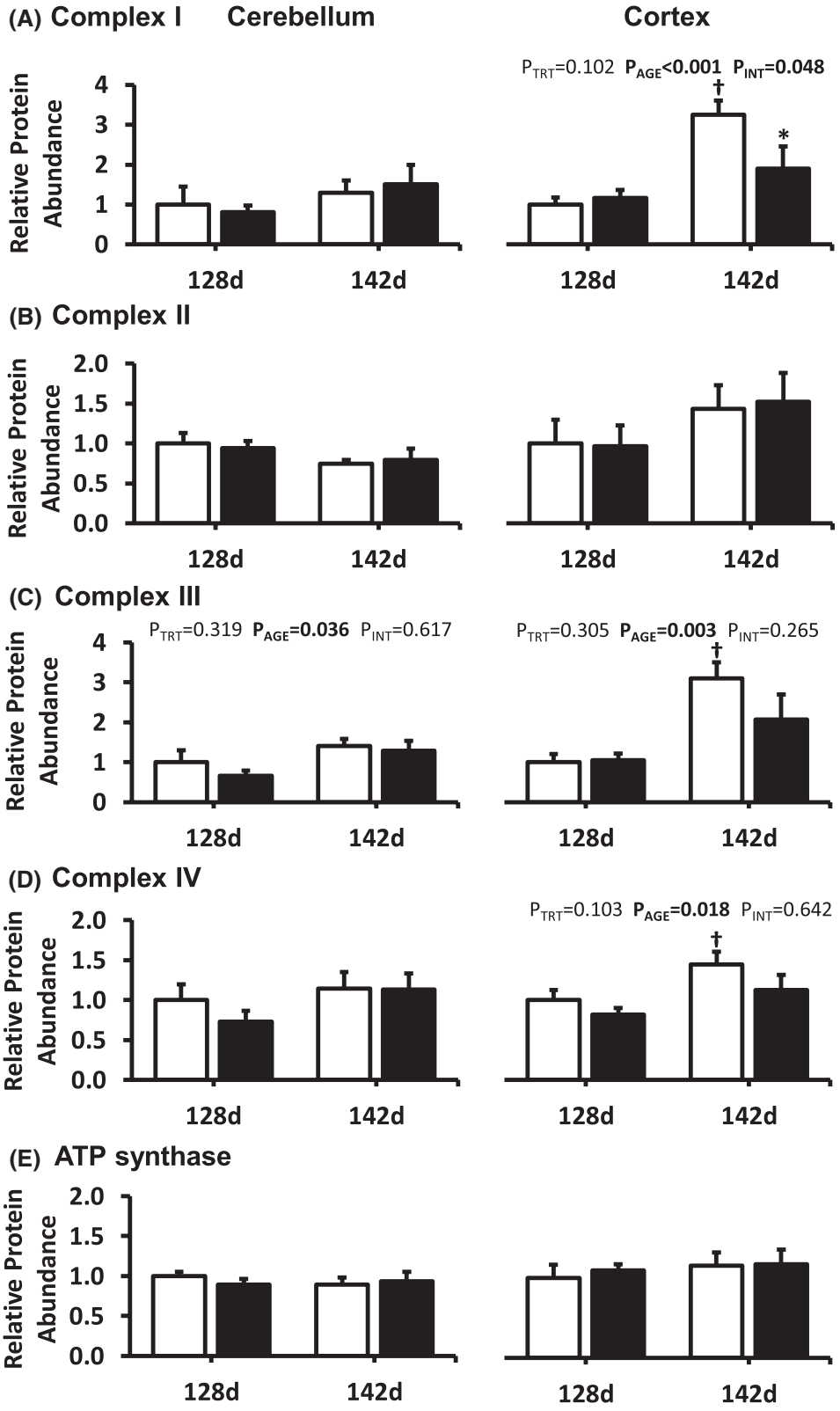

FIGURE 2 Mitochondrial ETS Complex abundance in the cerebellum and cortex and impact of hypothyroidism. Mean ± SEM protein abundance in the cerebellum and cortex at 128 and 142 days (d) gestation for control (open columns) and thyroidectomized (TX, filled columns) fetuses. Statistical significance following a two-way ANOVA examining the effects of treatment (P_{TRT}), age (P_{AGE}), and their interaction (P_{INT}) are presented above each figure when significant, *significantly different from control at the same gestational age, †significantly different from values at 128 dGA ($P < .05$, two-way ANOVA + Tukey's *post hoc* test per region). n = 5 per group

TABLE 2 Correlations and partial correlations of cerebellum and cortex mitochondrial parameters and citrate synthase activity with circulating T3 and cortisol

Cerebellum	**CI_L**	**CI_P**	**$CI\&CII_P$**	**CII_P**	**CS activity**
Correlations					
Log_{10} plasma cortisol (ng/mL)	$r = 0.109$	$r = 0.536$	$r = 0.643$	$r = 0.538$	$r = 0.275$
	$P = .620$	$P = .008$	$P = .001$	$P = .017$	$P = .216$
	n = 23	n = 23	n = 23	n = 19	n = 22
Log_{10} plasma T_3 (ng/mL)	$r = 0.073$	$r = 0.473$	$r = 0.498$	$r = 0.454$	$r = 0.119$
	$P = .743$	$P = .023$	$P = .016$	$P = .051$	$P = .597$
	n = 23	n = 23	n = 23	n = 19	n = 22
Partial correlations					
Log_{10} plasma cortisol (ng/mL)		$r = 0.368$	$r = 0.504$		
		$P = .092$	$P = .017$		
		n = 23	n = 23		
	NA			NA	NA
Log_{10} plasma T_3 (ng/mL)		$r = 0.242$	$r = 0.209$		
		$P = .278$	$P = .352$		
		n = 23	n = 23		
Cortex	**CI_L**	**CI_P**	**$CI\&CII_P$**	**CII_P**	**CS activity**
Correlations					
Log_{10} plasma cortisol (ng/mL)	$r = -0.053$	$r = 0.440$	$r = 0.403$	$r = 0.393$	$r = 0.608$
	$P = .788$	$P = .019$	$P = .033$	$P = .086$	$P = .001$
	n = 28	n = 28	n = 28	n = 20	n = 27
Log_{10} plasma T_3 (ng/mL)	$r = -0.152$	$r = 0.260$	$r = 0.215$	$r = 0.077$	$r = 0.391$
	$P = .440$	$P = .182$	$P = .272$	$P = .747$	$P = .044$
	n = 28	n = 28	n = 28	n = 20	n = 27
Partial correlations					
Log_{10} plasma cortisol (ng/mL)					$r = 0.507$
					$P = .008$
					n = 27
	NA	NA	NA	NA	
Log_{10} plasma T_3 (ng/mL)					$r = 0.023$
					$P = .910$
					n = 27

Note: Relationship between log10 plasma T_3 and cortisol data and CI-linked leak respiration (CI_L), CI- (CI_P), CI&II- ($CI\&CII_P$), and CII- (CII_P) linked OXPHOS capacity, and citrate synthase (CS) activity. Data presented for all animals from 104 to 142 days gestational age (dGA), control, and thyroidectomized (TX). NA, not analyzed.

cerebellar CI&CII-linked OXPHOS capacity (Table 2). In the cortex, Cl_P and $CI\&CII_P$ were correlated with cortisol but not to T_3 (Table 2). In both regions, there were no significant correlation between leak state respiration and either hormone concentration (Table 2). Cortical CS activity was correlated positively with both T_3 and cortisol concentrations, with cortisol the more dominant influence using partial correlation analysis (Table 2). There were no significant correlations between cerebellar CS activity and either hormone concentration (Table 2).

3.3 | Protein abundance of electron transfer system (ETS) complexes

Cortical abundance of ETS Complex I was significantly lower in TX than control fetuses at 142 dGA but not 128 dGA (Figures 2A and S2A). Fetal thyroidectomy also attenuated the normal increase in cortical abundance of Complexes I, III, and IV seen in the control fetuses toward term (Figures 2A,C,D and S2A,C,D). Hypothyroidism had no effect on the cerebellar abundance or developmental profile of Complexes I to IV (Figures 2A-D and

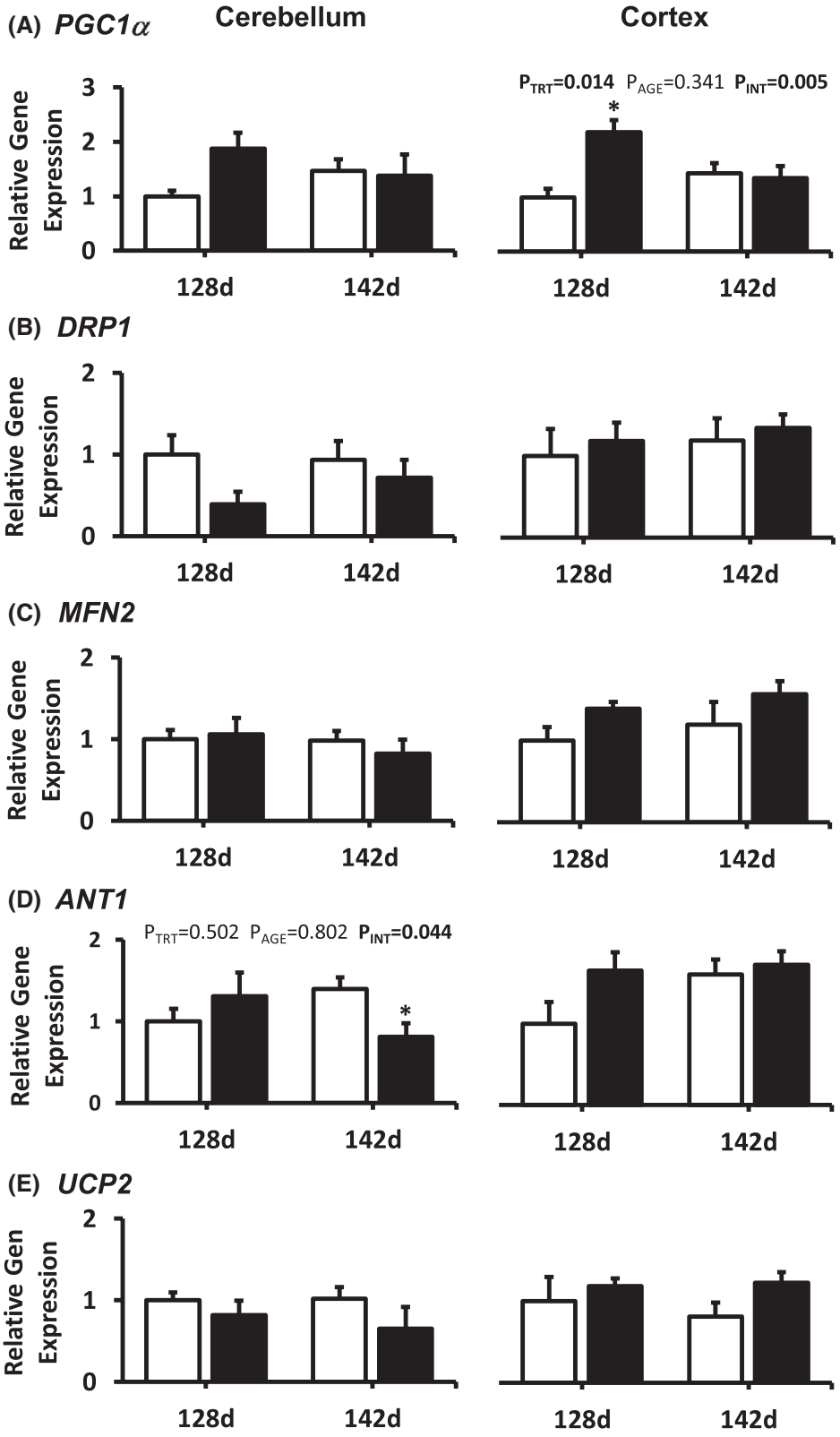

FIGURE 3 Expression of genes involved in mitochondrial biogenesis and dynamics in the cerebellum and cortex and impact of hypothyroidism. Mean ± SEM mRNA levels of (A) peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC1α*), (B) dynamin-related protein 1 (*DRP1*), (C) mitofusin 2 (*MFN2*), (D) adenine nucleotide translocase 1 (*ANT1*), and (E) uncoupling protein 2 (*UCP2*) in the cerebellum and cortex at 128 and 142 days (d) gestation for control (open columns) and thyroidectomized (TX, filled columns) fetuses. Statistical significance following a two-way ANOVA examining the effects of treatment (P_{TRT}), age (P_{AGE}), and their interaction (P_{INT}) are presented above each figure when significant, and * significantly different from control at the same gestational age ($P < .05$, two-way ANOVA + Tukey's *post hoc* test per region). n = 5-6 per group

S2A-D). In neither region was ATP-synthase abundance affected by hypothyroidism or age (Figures 2E and S2E).

3.4 | Expression of genes essential for mitochondrial bioenergetics and dynamics

Hypothyroidism affected cerebral expression of *PGC1α,* a major transcriptional regulator of mitochondrial biogenesis. Cortical *PGC1α* expression was significantly higher in TX than control fetuses at 128 dGA but not by 142 dGA (Figure 3A). The pattern of cerebellar *PGC1α* expression was similar, but did not reach statistical significance (Figure 3A, $P > .05$, all factors). In both brain regions, hypothyroidism had no significant effect on gene expression of *DRP1* and *MFN2*, the products of which support mitochondrial fission and fusion, respectively (Figure 3B,C).

There were regional and age-specific effects of hypothyroidism on gene expression of *ANT1* (Figure 3D). In the cerebellum, but not cortex, there was an interaction between treatment and age with lower *ANT1* expression in TX than control fetuses at 142 dGA (Figure 3D). Hypothyroidism had no effect on cerebellar or cortical *UCP2* expression at either age (Figure 3E).

3.5 | Thyroid hormone receptor beta (THRβ) and glucocorticoid receptor (GR) gene expression

Cortical *THRβ* expression increased between 128 dGA and 142 dGA in TX but not control fetuses, and was significantly higher in TX than control fetuses by 142 dGA (Figure 4A). No significant changes in cerebellar *THRβ* expression occurred with hypothyroidism or age (Figure 4A). Conversely, hypothyroidism reduced cerebellar but not cortical *GR* expression with the cerebellar effect primarily at 128 dGA (Figure 4B). Cerebellar *GR* expression also declined with age in control but not TX fetuses (Figure 4B). Cortical *GR* expression was not affected by age or thyroidectomy (Figure 4B).

3.6 | Cerebral morphology

At 104 dGA, immunostaining of myelin basic protein (MBP) was not present in sufficient quantities to be detectable by the imaging software in either brain region. Therefore, histological analyses were limited to 128 dGA and 142 dGA. In the cerebellum, hypothyroidism significantly reduced the total area of MBP staining at 142 dGA, but not 128 dGA, relative

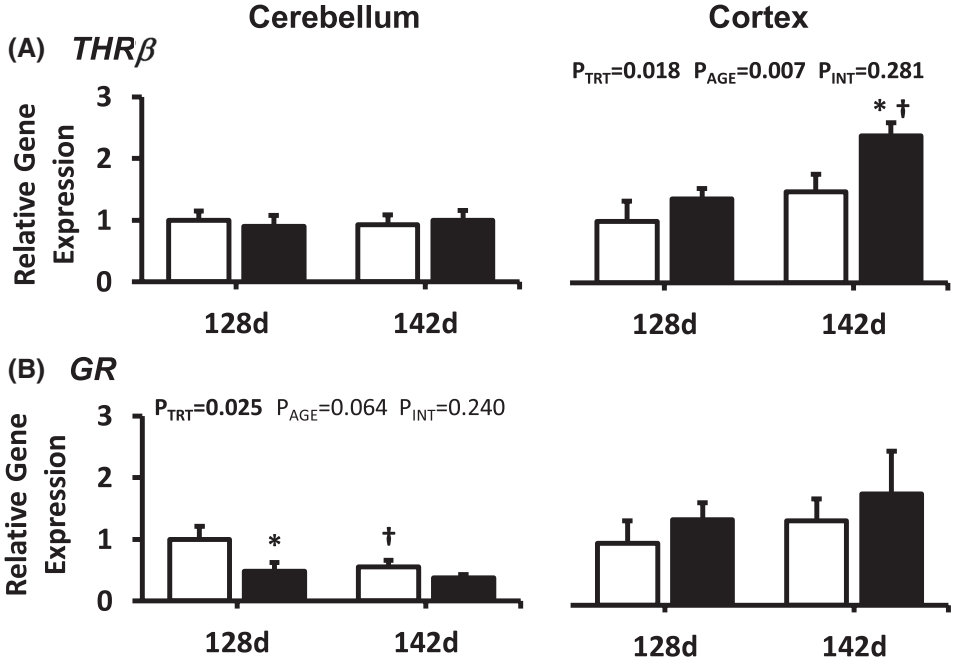

FIGURE 4 Thyroid hormone and glucocorticoid receptor gene expression in the cerebellum and cortex and impact of hypothyroidism. Mean ± SEM mRNA levels of (A) thyroid hormone receptor beta (*THRβ*) and (B) glucocorticoid receptor (*GR*) in the cerebellum and cortex at 128 and 142 days (d) gestational for control (open columns) and thyroidectomized (TX, filled columns) fetuses. Statistical output following a two-way ANOVA examining the effects of treatment (P_{TRT}), age (P_{AGE}), and their interaction (P_{INT}) are presented above each figure when significant, * significantly different from control at the same gestational age, and †significantly different from values at 128 dGA ($P < .05$, two-way ANOVA + Tukey's *post hoc* test per region). n = 5-6 per group

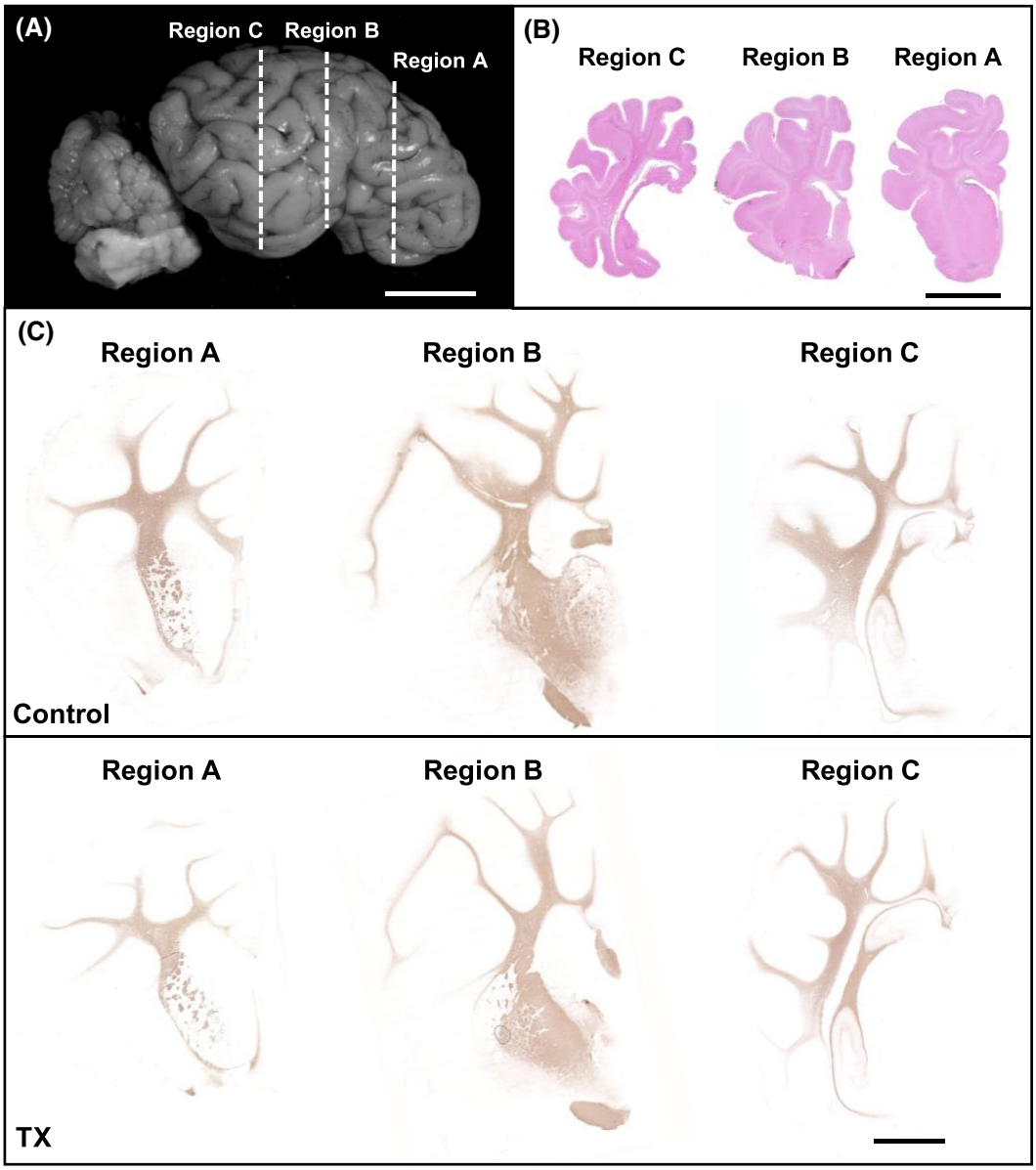

FIGURE 5 Cerebral morphology and myelination in the cerebrum and impact of hypothyroidism. Representative images showing (A) the level at which the brain regions were sectioned and stained with hematoxylin & eosin (H&E) and myelin basic protein (MBP) at 142 days of gestation (dGA), (B) H&E-stained sections showing the morphology of the three brain regions, and (C) MBP-positive immunostaining of the cerebrum from control and thyroidectomized (TX) fetuses at 142 dGA. Scale bar Figure A = 1 cm, Figure B = 10 mm, and Figure C = 5 mm

to controls (Area of myelin, Table 3). Total cerebellar area, the proportion of MBP-positive staining relative to total area (%), and optical density (OD) of MBP staining were unaffected by thyroidectomy irrespective of age (Table 3).

Hypothyroidism significantly reduced the total area of the caudal subregion of the cerebrum (level C) at 128 dGA and 142 dGA relative to control fetuses, but had no effect on the area of the other levels (Table 3 and Figure 5). The area of myelin (all levels), proportion of myelin (%, all levels), and the OD of periventricular (level A) and intragyral (all levels) myelin were all significantly less in TX than control fetuses at 142 dGA, and, in some instance, also at 128 dGA (Table 3 and Figure 5). The gyrification index of the cerebrum was not altered by thyroidectomy at any level (Table 3).

4 | DISCUSSION

This study demonstrates that development of mitochondrial respiratory function in the ovine brain is compromised by thyroid hormone deficiency before birth. Fetal thyroidectomy impaired cerebral mitochondrial OXPHOS and attenuated the increase in ETS complexes normally seen toward term. It also altered cerebral expression of key genes involved in

TABLE 3 Morphology of the brain

		128 dGA Control	128 dGA TX	142 dGA Control	142dGA TX	Statistical significance[#]		
	Cerebellum	4♀, 3♂	3♀, 3♂	3♀, 3♂	2♀, 4♂	Treatment	Age	Interaction
	Total area of cerebellum (mm^2)	149.4 ± 13.2	159.4 ± 26.4	189.1 ± 13.7	165.5 ± 12.0	$P = .688$	$P = .188$	$P = .327$
	Area of myelin (mm^2)	32.9 ± 3.4	40.7 ± 1.9	58.1 ± 5.7†	46.0 ± 3.5*	$P = .575$	$P = .001$	$P = .021$
	Myelin (%)	22.1 ± 1.9	26.9 ± 3.0	31.9 ± 5.3	28.3 ± 2.5	$P = .857$	$P = .103$	$P = .208$
	Myelin-arbour vitae (OD)	0.175 ± 0.006	0.181 ± 0.009	0.181 ± 0.003	0.171 ± 0.006	$P = .788$	$P = .759$	$P = .249$
	Cerebrum							
Region A	Total area of cerebrum (mm^2)	262.9 ± 14.2	247.3 ± 11.3	258.2 ± 12.2	252.3 ± 10.9	$P = .404$	$P = .991$	$P = .702$
	Area of myelin (mm^2)	30.6 ± 1.7	22.3 ± 3.4	68.6 ± 2.4†	45.5 ± 5.7* †	$P = .001$	$P < .001$	$P = .055$
	Myelin (%)	11.8 ± 0.8	9.2 ± 1.5	26.7 ± 1.1†	17.9 ± 1.9* †	$P = .001$	$P < .001$	$P = .040$
	Myelin-periventricular (OD)	0.089 ± 0.009	0.068 ± 0.011	0.201 ± 0.007†	0.169 ± 0.007* †	$P = .006$	$P < .001$	$P = .506$
	Myelin-intragyral (OD)	0.057 ± 0.007	0.045 ± 0.009	0.183 ± 0.013†	0.130 ± 0.004* †	$P = .001$	$P < .001$	$P = .026$
	Gyrification index	2.23 ± 0.11	2.27 ± 0.11	2.17 ± 0.10	2.13 ± 0.10	$P = .977$	$P = .331$	$P = .714$
Region B	Total area of cerebrum (mm^2)	351.0 ± 11.1	331.7 ± 10.8	356.3 ± 15.4	327.3 ± 13.5	$P = .074$	$P = .971$	$P = .709$
	Area of myelin (mm^2)	86.6 ± 8.7	75.1 ± 8.0	142.1 ± 11.9†	96.2 ± 6.4*	$P = .004$	$P < .001$	$P = .065$
	Myelin (%)	24.5 ± 1.9	22.7 ± 2.5	39.7 ± 1.8†	29.6 ± 1.8* †	$P = .017$	$P < .001$	$P = .085$
	Myelin-periventricular (OD)	0.125 ± 0.008	0.114 ± 0.011	0.203 ± 0.005†	0.198 ± 0.018†	$P = .366$	$P < .001$	$P = .705$
	Myelin-intragyral (OD)	0.063 ± 0.008	0.037 ± 0.005*	0.184 ± 0.009†	0.140 ± 0.012* †	$P < .001$	$P < .001$	$P = .300$
	Gyrification index	2.18 ± 0.08	2.33 ± 0.10	2.15 ± 0.13	2.18 ± 0.07	$P = .352$	$P = .336$	$P = .502$
Region C	Total area of cerebrum (mm^2)	265.9 ± 7.9	231.4 ± 14.5*	286.6 ± 16.9	239.5 ± 6.4*	$P = .002$	$P = .220$	$P = .585$
	Area of myelin (mm^2)	35.5 ± 4.2	18.3 ± 4.6*	77.7 ± 2.9†	44.4 ± 3.9* †	$P < .001$	$P < .001$	$P = .094$
	Myelin (%)	13.6 ± 1.9	7.6 ± 1.4*	27.4 ± 2.5†	18.5 ± 1.3* †	$P = .001$	$P < .001$	$P = .450$
	Myelin-periventricular (OD)	0.100 ± 0.010	0.085 ± 0.015	0.219 ± 0.005†	0.199 ± 0.018†	$P = .208$	$P < .001$	$P = .828$
	Myelin-intragyral (OD)	0.066 ± 0.009	0.035 ± 0.009*	0.208 ± 0.005†	0.156 ± 0.009* †	$P < .001$	$P < .001$	$P = .247$
	Gyrification index	2.10 ± 0.09	2.19 ± 0.04	2.11 ± 0.15	2.07 ± 0.05	$P = .734$	$P = .497$	$P = .438$

Note: Data are presented as mean±SEM at 128 and 142 days of gestational age (dGA) for control and thyroidectomized (TX) fetuses.

[#]Statistical significance for the comparison of control and TX data by two-way ANOVA with treatment and age as factors plus their interaction; *significantly different from control at the same gestational age, and; †significantly different from value at 128 dGA ($P < .05$ by two-way ANOVA + Tukey's *post hoc* test).

TABLE 4 Summary of the effects of thyroidectomy on cerebral mitochondria, hormone receptors, and morphology

	Cerebellum	Cortex/Cerebrum
Mitochondria		
OXPHOS respiratory rates	↓ CI&CII$_P$	↓ CI$_L$ at 128 dGA
	↓ CII$_P$	↓ CI$_P$ at 128 dGA
	Attenuated ↑ toward term	Delayed ↑ toward term
ETS complexes	↔ with TX or age	↓ Complex I at 142 dGA
		Attenuated ↑ in Complexes I, II, & IV toward term
Biogenesis and dynamics	↔ PGC1α expression	↑ PGC1α expression at 128 dGA
	↓ ANT1 expression at 142 dGA	↔ ANT1 expression
Hormone receptors		
THRβ expression	↔ with TX or age	↑ at 142 dGA
GR expression	↓ and attenuated ↓ with age	↔ with TX or age
Morphology		
Size	↔	↓ region specific with age
Myelination	↓ at 142 dGA	↓ region specific with age

Note: ↓ = decrease, ↑ = increase, and ↔ = no change; adenine nucleotide translocase (ANT1), days of gestational age, (dGA), and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α).

mitochondrial biogenesis and ATP production, as well as the expression of receptors for the thyroid and glucocorticoid hormones known to be essential for prepartum maturation of many other metabolic processes.[6] The effects of hypothyroidism on cerebral mitochondrial function were dependent on gestational age and differed between the cerebellum and cortex. They were also accompanied by regional changes in brain morphology and significant reductions in myelination. The changes in cerebral mitochondrial function, hormone receptors, and morphology induced by thyroidectomy are summarized in Table 4 for the two brain regions. Collectively, the findings highlight the importance of thyroid hormone signaling in the prepartum preparation of cerebral mitochondria for the increased energy demands associated with the transition from intra- to extra-uterine life.

4.1 | Hypothyroidism and regional rates of cerebral mitochondrial respiratory function

Hypothyroidism reduced mitochondrial OXPHOS capacity in both the cerebellum and cortex with different age-related patterns. In the cerebellum, fetal thyroidectomy reduced CI&II OXPHOS capacity overall, in association with impaired CII-linked respiration. However, thyroid hormone deficiency had no effect on the cerebellar abundance of Complex II or any of the other ETS complexes. In contrast, in the hypothyroid cortex, the decrease in OXPHOS capacity was primarily related to reduced respiration with CI-linked substrates, predominantly at 128 dGA. Hypothyroidism had no effect on CII-linked respiration in the cortex in the current study, or in a previous study of hypothyroid neonatal rats.[25] Consistent with the present respiratory findings, Complex I but not Complex II abundance was reduced in the cortex of TX fetuses compared with controls by 142 dGA. Similarly, previous studies have shown lower Complex I abundance and rates of OXPHOS from NADH-generating substrates in the cortex but not the cerebellum of hypothyroid neonatal rats.[15] Certainly, in the current study, the flux control ratios suggest that, in both brain regions, there is relative protection of CII- over CI-linked respiration in the face of an overall reduction in OXPHOS capacity after fetal thyroidectomy.

4.2 | Hypothyroidism and cerebral mitochondrial biogenesis and OXPHOS efficiency

In both hypothyroid brain regions, the reduced OXPHOS capacity was not accompanied by any significant change in mitochondrial density measured by CS activity. However, OXPHOS capacity can be altered by other mitochondrial characteristics, such as morphology and substrate transport without any change in mitochondrial density.[16] In hypothyroid newborn rats, cerebral mitochondria have a reduced number of abnormally shaped cristae, in association with decreased mitochondrial respiratory rates compared to controls.[15,25-28] These changes were region-specific and tended to be more pronounced in the cortex than other brain regions.[25,26] In the current study, cortical *PGC1α* expression was increased in

TX fetuses, which may suggest a compensatory response to the reduced OXPHOS capacity. However, the lack of any change in cortical *DRP1* and *MFN2* expression may have compromised mitochondrial membrane dynamics, thereby reducing respiratory activity.[13,16] There were no significant changes in ATP synthase abundance, *UCP1* expression, or in the OXPHOS coupling efficiency in either hypothyroid brain region, which suggests there was no modification in mitochondrial respiration efficiency. Consequently, the explanation for the reduced cerebral mitochondrial respiration in hypothyroid conditions is likely to differ regionally and may relate to the lower Complex I abundance per mitochondrial unit in the cortex but reflect the lower *ANT1* expression reducing ADP availability for OXPHOS in the cerebellum near term. Previous studies in adult rats and newborn sheep have shown that thyroid hormone deficiency reduces ANTI abundance in a tissue-specific manner in relation to mitochondrial OXPHOS capacity.[29-31]

4.3 | Hypothyroidism and the developmental profile of mitochondrial OXPHOS capacity toward term

Cerebral upregulation of mitochondrial OXPHOS capacity in control fetuses near team was attenuated in their hypothyroid twins with reduced or delayed rises in OXPHOS respiratory rates and lower cortical expression of several ETS complexes by 142 dGA. These ontogenic changes in mitochondrial development may have been due, in part, to the lower cortisol concentrations after thyroidectomy in this and previous studies.[24] Glucocorticoids are known to increase cortical OXPHOS capacity and Complex IV expression in the liver and kidney of fetal rats during late gestation.[32,33] Similarly, in fetal sheep, the prepartum increase in OXPHOS capacity and ETS complex expression in skeletal muscle closely parallels the normal prepartum rise in cortisol concentrations.[13] In the present study, the concentration of cortisol appeared to be a significant influence on cerebral OXPHOS capacity in addition to T_3 availability. Cortisol is known to be responsible for the normal prepartum rise in plasma T_3 in fetal sheep by activating the tissue deiodinases that convert T_4 into T_3 peripherally.[34] Consequently, the normal upregulation of cerebral OXPHOS capacity toward term may be a cortisol-dependent, T_3-mediated process of maturation as seen with other metabolic functions essential for neonatal survival.[6]

The differences in *THRβ* and *GR* expression with gestational age and between the TX and control fetuses may also contribute to the normal prepartum increase in cerebral OXPHOS capacity and to the changes seen in this developmental profile in hypothyroid fetuses. More specifically, the low cerebellar *GR* expression of TX fetuses may further limit any maturational effects of the low cortisol levels on OXPHOS capacity toward term, thereby contributing to the reduced $CI\&CII_P$ and CII_P respiratory rates seen in these fetuses. In contrast, the upregulated cortical *THRβ* expression in TX fetuses at 142 dGA may assist with restoring the normal rate of CI-linked respiration, despite the lower T_3 and cortisol concentrations.

4.4 | Hypothyroidism and cerebral morphology

Hypothyroidism *in utero* had both regional and temporal effects on cerebral size and myelination, which were more pronounced and occurred earlier in gestation in the cortex than cerebellum. This contrasts with the significant alterations in cerebellar morphology and myelination seen in hypothyroid neonatal rats at similar stages of brain development.[23] Thyroid hormones are known to regulate the differentiation and proliferation of oligodendrocytes responsible for axon myelination, but have little effect on the generation or total number of axons per se.[35-40] Thyroid hormones has also been shown to influence oligodendrocyte expression of myelin-producing genes including MBP.[41-44] Thus, the reduction in mature myelin in both the cerebrum and cerebellum of TX fetuses may be due to reductions in the generation and/or myelin production of oligodendrocytes. Previous rodent studies have suggested that myelin genes are sensitive to thyroid hormones only at the onset of myelination, and not when the process is complete.[23] This may explain the greater cortical effect of hypothyroidism in the current study, as myelination begins later in the cortex than cerebellum in fetal sheep, and may have already been near completion in the cerebellum at the time of fetal thyroidectomy.[45,46] Whatever the specific mechanisms involved, development of the cell populations in the hypothyroid brain are likely to have been compromised by lower ATP availability as a result of the reduced mitochondrial OXPHOS capacity.

In summary, the current study is the first to demonstrate the effects of hypothyroidism on cerebral mitochondrial function in an animal with a similar thyroid hormone environment and pattern of brain development to human infants. Fetal hypothyroidism impaired cerebral mitochondrial OXPHOS, ETS complex abundance, and the expression of key genes involved in mitochondrial biogenesis and ATP availability. These effects were regional and temporal, and appeared to relate, in part, to the concomitant impairment of pituitary-adrenal function in hypothyroid conditions.[24] While further studies are needed to identify the specific molecular pathways involved in endocrine regulation of cerebral mitochondrial function, the current findings have important implications for the etiology and treatment of the neurodevelopmental abnormalities seen in human infants born prematurely or with congenital hypothyroidism.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

A.L. Fowden and E.J. Camm: Conceptualization; K.L. Davies and E.J. Camm: Data curation; K.L. Davies, D.J. Smith, and E.J. Camm: Formal analysis; A.J. Murray and A.L. Fowden: Funding acquisition; K.L. Davies, D.J. Smith, M.E. Stewart, A. Easwaran, P.F.P. Wooding, A.J. Forhead, A.L. Fowden, and E.J. Camm: Investigation; K.L. Davies, T. El-Bacha, A.J. Forhead, A.J. Murray, A.L. Fowden, and E.J. Camm: Methodology; K.L. Davies, A.L. Fowden and E.J. Camm: Project administration; A.J. Murray and A.L. Fowden: Resources; K.L. Davies, A.L. Fowden, and E.J. Camm: Supervision; T. El-Bacha, A.L. Fowden, and E.J. Camm: Validation; K.L. Davies, T. El-Bacha., A.J. Forhead, A.J. Murray, A.L. Fowden, and E.J. Camm: Writing-Original Draft, Review, and Editing.

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REFERENCES

1. Wassner AJ. Congenital hypothyroidism. *Clin Perinatol*. 2018;45(1):1-18. https://doi.org/10.1016/j.clp.2017.10.004.
2. Prezioso G, Giannini C, Chiarelli F. Effect of thyroid hormones on neurons and neurodevelopment. *Horm Res Paediatr*. 2018;90(2):73-81. https://doi.org/10.1159/000492129.
3. Mannisto T, Mendola P, Reddy U, Laughon SK. Neonatal outcomes and birth weight in pregnancies complicated by maternal thyroid disease. *Am J Epidemiol*. 2013;178(5):731-740. https://doi.org/10.1093/aje/kwt031.
4. Léger J, Olivieri A, Donaldson M, et al. European Society for Paediatric Endocrinology consensus guidelines on screening, diagnosis, and management of congenital hypothyroidism. *J Clin Endocrinol Metab*. 2014;99(2):363-384. https://doi.org/10.1210/jc.2013-1891.
5. Batistuzzo A, Ribeiro MO. Clinical and subclinical maternal hypothyroidism and their effects on neurodevelopment, behavior and cognition. *Arch Endocrinol Metab*. 2020;64(1):89-95. https://doi.org/10.20945/2359-3997000000201.
6. Forhead AJ, Fowden AL. Thyroid hormones in fetal growth and prepartum maturation. *J Endocrinol*. 2014;221(3):R87-R103. https://doi.org/10.1530/JOE-14-0025.
7. McIntosh GH, Potter BJ, Hetzel BS, Hua CH, Cragg BG. The effects of 98 day foetal thyroidectomy on brain development in the sheep. *J Comp Pathol*. 1982;92(4):599-607. https://doi.org/10.1016/0021-9975(82)90012-3.
8. Lopez M, Alvarez CV, Nogueiras R, Dieguez C. Energy balance regulation by thyroid hormones at central level. *Trends Mol Med*. 2013;19(7):418-427. https://doi.org/10.1016/j.molmed.2013.04.004.
9. Dawes GS, Mott JC. The increase in oxygen consumption of the lamb after birth. *J Physiol*. 1959;146(2):295-315. https://doi.org/10.1113/jphysiol.1959.sp006194.
10. Breall JA, Rudolph AM, Heymann MA. Role of thyroid hormone in postnatal circulatory and metabolic adjustments. *J Clin Invest*. 1984;73(5):1418-1424. https://doi.org/10.1172/JCI111346.
11. Polk DH, Callegari CC, Newnham J, et al. Effect of fetal thyroidectomy on newborn thermogenesis in lambs. *Pediatr Res*. 1987;21(5):453-457. https://doi.org/10.1203/00006450-198705000-00006.
12. Herpin P, Berthon D, Duchamp C, Dauncey MJ, Le Dividich J. Effect of thyroid status in the perinatal period on oxidative capacities and mitochondrial respiration in porcine liver and skeletal muscle. *Reprod Fertil Dev*. 1996;8(1):147-155. https://doi.org/10.1071/rd9960147.
13. Davies KL, Camm EJ, Atkinson EV, et al. Development and thyroid hormone dependence of skeletal muscle mitochondrial function towards birth. *J Physiol*. 2020;598(12):2453-2468. https://doi.org/10.1113/JP279194.
14. Harris SE, De Blasio MJ, Zhao X, et al. Thyroid deficiency before birth alters the adipose transcriptome to promote overgrowth of white adipose tissue and impair thermogenic capacity. *Thyroid*. 2020;30(6):794-805. https://doi.org/10.1089/thy.2019.0749.
15. Martinez B, del Hoyo P, Martin MA, Arenas J, Perez-Castillo A, Santos A. Thyroid hormone regulates oxidative phosphorylation in the cerebral cortex and striatum of neonatal rats. *J Neurochem*. 2001;78(5):1054-1063. https://doi.org/10.1046/j.1471-4159.2001.00487.x.
16. Hagberg H, Mallard C, Rousset CI, Thornton C. Mitochondria: hub of injury responses in the developing brain. *Lancet Neurol*. 2014;13(2):217-232. https://doi.org/10.1016/S1474-4422(13)70261-8.
17. Brand M, Pakay J, Ocloo A, et al. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem J*. 2005;392(Pt 2):353-362. https://doi.org/10.1042/BJ20050890.
18. Lombardi A, Moreno M, de Lange P, Iossa S, Busiello RA, Goglia F. Regulation of skeletal muscle mitochondrial activity by thyroid hormones: focus on the "old" triiodothyronine and the "emerging" 3,5-diiodothyronine. *Front Physiol*. 2015;6:237. https://doi.org/10.3389/fphys.2015.00237.
19. Gnanalingham MG, Mostyn A, Symonds ME, Stephenson T. Ontogeny and nutritional programming of adiposity in sheep: potential role of glucocorticoid action and uncoupling protein-2. *Am*

J Physiol Regul Integr Comp Physiol. 2005;289(5):R1407-R1415. https://doi.org/10.1152/ajpregu.00375.2005.

20. Freitas TP, Rezin GT, Gonçalves CL, et al. Evaluation of citrate synthase activity in brain of rats submitted to an animal model of mania induced by ouabain. *Mol Cell Biochem*. 2010;341(1-2):245-249. https://doi.org/10.1007/s11010-010-0455-0.
21. Larsen S, Nielsen J, Hansen CN, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol*. 2012;590(14):3349-3360. https://doi.org/10.1113/jphysiol.2012.230185.
22. Burtscher J, Zangrandi L, Schwarzer C, Gnaiger E. Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high-resolution respirometry. *Mitochondrion*. 2015;25:104-112. https://doi.org/10.1016/j.mito.2015.10.007.
23. Bernal J. Action of thyroid hormone in brain. *J Endocrinol Invest*. 2002;25(3):268-288. https://doi.org/10.1007/BF03344003.
24. Camm EJ, Inzani I, De Blasio MJ, et al. Thyroid hormone deficiency suppresses fetal pituitary-adrenal function near term: implications for the control of fetal maturation and parturition. *Thyroid*. 2020;https://doi.org/10.1089/thy.2020.0534.
25. Martinez B, Rodrigues TB, Gine E, Kaninda JP, Perez-Castillo A, Santos A. Hypothyroidism decreases the biogenesis in free mitochondria and neuronal oxygen consumption in the cerebral cortex of developing rats. *Endocrinology*. 2009;150(8):3953-3959. https://doi.org/10.1210/en.2008-1755.
26. Vega-Nunez E, Alvarez AM, Menendez-Hurtado A, Santos A, Perez-Castillo A. Neuronal mitochondrial morphology and transmembrane potential are severely altered by hypothyroidism during rat brain development. *Endocrinology*. 1997;138(9):3771-3778. https://doi.org/10.1210/endo.138.9.5407.
27. Gadaleta MN, Renis M, Minervini GR, et al. Effect of hypothyroidism on the biogenesis of free mitochondria in the cerebral hemispheres and in cerebellum of rat during postnatal development. *Neurochem Res*. 1985;10(2):163-177. https://doi.org/10.1007/BF00964565.
28. Rajan RR, Katyare SS. Effect of 3,5,3′-tri-iodothyronine on cellular growth and oxygen consumption in neonatal rat brain. *Experientia*. 1982;38(9):1110-1114. https://doi.org/10.1007/BF01955395.
29. Sterling K, Brenner MA. Thyroid hormone action: effect of triiodothyronine on mitochondrial adenine nucleotide translocase in vivo and in vitro. *Metabolism*. 1995;44(2):193-199. https://doi.org/10.1016/0026-0495(95)90264-3.
30. Bobyleva V, Bellei M, Pazienza TL, Muscatello U. Effect of cardiolipin on functional properties of isolated rat liver mitochondria. *Biochem Mol Biol Int*. 1997;41(3):469-480. https://doi.org/10.1080/15216549700201491.
31. Portman MA, Xiao Y, Qian K, Tucker RL, Parish SM, Ning XH. Thyroid hormone coordinates respiratory control maturation and adenine nucleotide translocator expression in heart in vivo. *Circulation*. 2000;102(11):1323-1329. https://doi.org/10.1161/01.cir.102.11.1323.
32. Nakai A, Shibazaki Y, Taniuchi Y, et al. Effect of dexamethasone on mitochondrial maturation in the fetal rat brain. *Am J Obstet Gynecol*. 2002;186(3):574-578. https://doi.org/10.1067/mob.2002.121542.
33. Prieur B, Bismuth J, Delaval E. Effects of adrenal steroid hormones on mitochondrial maturation during the late fetal period. *Eur J Biochem*. 1998;252(2):194-199. https://doi.org/10.1046/j.1432-1327.1998.2520194.x.
34. Forhead AJ, Curtis K, Kaptein E, Visser TJ, Fowden AL. Developmental control of iodothyronine deiodinases by cortisol in the ovine fetus and placenta near term. *Endocrinology*. 2006;147(12):5988-5994. https://doi.org/10.1210/en.2006-0712.
35. Jones SA, Jolson DM, Cuta KK, Mariash CN, Anderson GW. Triiodothyronine is a survival factor for developing oligodendrocytes. *Mol Cell Endocrinol*. 2003;199(1-2):49-60. https://doi.org/10.1016/s0303-7207(02)00296-4.
36. Durand B, Raff M. A cell-intrinsic timer that operates during oligodendrocyte development. *BioEssays*. 2000;22(1):64-71. https://doi.org/10.1002/(SICI)1521-1878(200001)22:1<64:AID-BIES11>3.0.CO;2-Q.
37. Dugas JC, Ibrahim A, Barres BA. The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. *Mol Cell Neurosci*. 2012;50(1):45-57. https://doi.org/10.1016/j.mcn.2012.03.007.
38. Barres BA, Lazar MA, Raff MC. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development*. 1994;120(5):1097-1108.
39. Gravel C, Sasseville R, Hawkes R. Maturation of the corpus callosum of the rat: II. Influence of thyroid hormones on the number and maturation of axons. *J Comp Neurol*. 1990;291(1):147-161. https://doi.org/10.1002/cne.902910110.
40. Berbel P, Guadano-Ferraz A, Angulo A, Ramon CJ. Role of thyroid hormones in the maturation of interhemispheric connections in rats. *Behav Brain Res*. 1994;64(1-2):9-14. https://doi.org/10.1016/0166-4328(94)90114-7.
41. Jeannin E, Robyr D, Desvergne B. Transcriptional regulatory patterns of the myelin basic protein and malic enzyme genes by the thyroid hormone receptors alpha1 and beta1. *J Biol Chem*. 1998;273(37):24239-24248. https://doi.org/10.1074/jbc.273.37.24239.
42. Farsetti A, Mitsuhashi T, Desvergne B, Robbins J, Nikodem VM. Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. *J Biol Chem*. 1991;266(34):23226-23232.
43. Farsetti A, Desvergne B, Hallenbeck P, Robbins J, Nikodem VM. Characterization of myelin basic protein thyroid hormone response element and its function in the context of native and heterologous promoter. *J Biol Chem*. 1992;267(22):15784-15788.
44. Tosic M, Torch S, Comte V, Dolivo M, Honegger P, Matthieu JM. Triiodothyronine has diverse and multiple stimulating effects on expression of the major myelin protein genes. *J Neurochem*. 1992;59(5):1770-1777. https://doi.org/10.1111/j.1471-4159.1992.tb11009.x.
45. Barlow RM. The foetal sheep: morphogenesis of the nervous system and histochemical aspects of myelination. *J Comp Neurol*. 1969;135(3):249-262. https://doi.org/10.1002/cne.901350302.
46. Back SA, Riddle A, Hohimer AR. Role of instrumented fetal sheep preparations in defining the pathogenesis of human periventricular white-matter injury. *J Child Neurol*. 2006;21(7):582-589. https://doi.org/10.1177/08830738060210070101.
47. Myers DA, Hanson K, Mlynarczyk M, Kaushal KM, Ducsay CA. Long-term hypoxia modulates expression of key genes regulating adipose function in the late-gestation ovine fetus. *Am J Physiol Regul Integr Comp Physiol*. 2008;294(4):R1312-R1318. https://doi.org/10.1152/ajpregu.00004.2008.

48. Kelly AK, Waters SM, McGee M, Fonseca RG, Carberry C, Kenny DA. mRNA expression of genes regulating oxidative phosphorylation in the muscle of beef cattle divergently ranked on residual feed intake. *Physiol Genomics*. 2011;43(1):12-23. https://doi.org/10.1152/physiolgenomics.00213.2009.
49. Reddy PH, Manczak M, Yin X, et al. Protective effects of a natural product, curcumin, against amyloid beta induced mitochondrial and synaptic toxicities in Alzheimer's disease. *J Investig Med*. 2016;64(8):1220-1234. https://doi.org/10.1136/jim-2016-000240.
50. Byrne K, Vuocolo T, Gondro C, et al. A gene network switch enhances the oxidative capacity of ovine skeletal muscle during late fetal development. *BMC Genomics*. 2010;11(1):378. https://doi.org/10.1186/1471-2164-11-378.
51. Tian YZ, Di J, Huang XX, et al. Selection of housekeeping genes for real-time fluorescence quantitative RT-PCR in skin of fine-wool sheep. *Asian J Anim Vet Adv*. 2013;8:473-482.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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